



Allergen stabilities and compatibilities in mixtures of high-protease fungal and insect extracts

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ABSTRACT

Background: Current practice guidelines state that protease-rich fungal and insect extracts can be combined when preparing immunotherapy vaccines, but data supporting the stability of allergens in these mixtures have not been reported.

Objective: To determine the stabilities and compatibilities of *Alternaria alternata* and German cockroach allergens in mixtures with other high-protease fungal and insect (cockroach, imported fire ant) extracts at final extract concentrations consistent with injection dose targets for maintenance immunotherapy.

Methods: Mixtures containing *Alternaria*, German cockroach, and other fungal and insect extracts frequently included in immunotherapy vaccines were analyzed by a combination of quantitative analyses (enzyme-linked immunosorbent assays for multiallergen immunoglobulin E [IgE]-binding potency, major *Alternaria* allergen Alt a 1, and major German cockroach allergens Bla g 1 and Bla g 2) and qualitative methods (immunoblotting). Mixtures and analogous single-extract controls containing 10 to 50% glycerin were evaluated after storage for up to 12 months at 2°C to 8°C.

Results: Mixtures of extracts within the same phylogenetic groups (fungal-fungal, insect-insect) retained favorable *Alternaria* and German cockroach allergen levels and activities under most conditions examined. For several cross-taxonomic (fungal-insect) extract combinations at 10 to 25% glycerin concentrations, different immunochemical test methods measuring single (major) or multiple allergens yielded threefold to 10-fold variations in allergen recoveries.

Conclusion: Allergen compatibilities can be compromised in some fungal-insect extract mixtures, contrary to current immunotherapy practice parameter recommendations. Separation of these products into different treatment vials may be required to produce stable mixtures for subcutaneous immunotherapy. Data from assay methodologies with distinct binding specificities provide a critical assessment of allergen activities in high-protease extract mixtures.

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Introduction

Subcutaneous allergen immunotherapy (SCIT) is a specific disease-modifying practice that can provide long-term benefits to many patients whose allergic symptoms cannot be controlled effectively by avoidance measures and pharmacotherapy.¹ Most patients undergoing SCIT are injected with complex mixtures of allergenic extracts derived from source materials that correspond closely to their clinical sensitivities. Maintenance concentrates are formulated with the intent to deliver consistent and effective allergen doses throughout the course of treatment.^{2,3}

One factor critical to optimization of SCIT mixtures is the potential degradative impact of active hydrolytic enzymes present in commercial extracts of numerous whole-body fungi and insects.

These enzymes can compromise the structures and antibody-binding activities of allergenic proteins from standardized pollen and dust mite extracts when mixed together in SCIT vaccines.^{4–6} Based on these findings, preparation of separate patient vials for high-protease (fungal, insect) and low-protease or protease-susceptible (pollen, mite, animal) extract mixtures has been recommended in previous and current SCIT practice parameter updates.²

Published studies from our laboratory and others have focused on the stability of target allergens in low-protease extracts after mixing and storage with other low-protease or high-protease products.^{6–10} No data have been reported on the mixing compatibilities of high-protease extract combinations recommended in the practice parameters and used regularly in many allergy clinics. To address this subject, in-house reference reagents for prominent fungal (*Alternaria alternata*) and insect (German cockroach, *Blattella germanica*) allergens were developed to support validation of allergen-specific quantitative immunoassays capable of detecting changes in individual (major) allergen concentrations and total

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extract (cumulative, multi-allergen) IgE-binding potencies. *In vitro* assays for specific allergens such as *Alternaria* Alt a 1 or German cockroach Bla g 1 measure epitope structures recognized by mouse or rabbit IgG antibodies, which may be different from those bound by IgE molecules in sensitive patients. Conversely, IgE-binding, inhibition-based potency assays involve numerous allergens that may or may not include the same structures detected in specific allergen tests. Using a combination of these methods, the present study was designed to assess the recoveries of *Alternaria* and German cockroach allergens after mixing with other fungal or insect extracts at final extract and glycerin concentrations typical of those employed for maintenance SCIT injections.

Methods

Allergenic extracts

The following nonstandardized extract concentrates at 1:20 w/v in 50% glycerin were obtained from Greer Laboratories: *Alternaria alternata*, *Aspergillus fumigatus*, *Penicillium chrysogenum* (notatum), *Bipolaris sorokiniana*, *Cladosporium sphaerospermum*, American cockroach (*Periplaneta americana*), German cockroach (*Blattella germanica*), and fire ant (*Solenopsis invicta*). Extracts were stored at 2°C to 8°C, and all product lots were mixed and tested within their expiration dates.

Extract mixtures and controls

Two-component extract mixtures and single-component controls (5.0-mL volumes) were formulated with each extract at one-tenth of concentrate levels, and analyzed after storage for up to 12 months at 2°C to 8°C. Final glycerin concentrations of these solutions were adjusted to 10%, 25%, and 50% v:v by addition of nonglycerinated and 50% glycerinated saline solutions containing 0.4% phenol (Greer Laboratories, Lenoir, North Carolina). Recoveries of *Alternaria* or German cockroach allergens in these solutions were determined and expressed relative to fresh target extract dilutions (immunoglobulin E [IgE] potency) or analogous single-extract control samples prepared and stored under the identical conditions as the extract mixtures (specific allergen concentrations).

Human sera

Freeze-dried human serum pools containing specific IgE to *Alternaria* allergens (Greer Laboratories lot HAM1-1) or German cockroach allergens (Greer Laboratories lot ZE-P7) were employed for human IgE enzyme-linked immunosorbent assay (ELISA) and immunoblot analyses.

Analytical methods

Human IgE ELISA inhibition assays were performed as described previously, using IgE-positive serum pools and glycerinated *Alternaria* or German cockroach extracts as coating and reference reagents.^{7,8} Microtiter plates, coating buffer, wash buffer, and detection reagents were identical to those reported in earlier studies. Absorbance values were determined at 405 nm in a Molecular Devices microplate reader. Parallelism and validity of test and reference curves were confirmed by paired *t* tests, and the relative IgE-binding potencies of test samples were determined by parallel line bioassay.

Alt a 1-specific double-bind ELISA analyses were conducted using rabbit anti-Alt a 1 capture and biotinylated probe antibodies, and Alt a 1 standard (1,000 ng/mL) obtained from Indoor Biotechnologies (Charlottesville, Virginia). Microtiter plates (Corning Inc., Corning, New York) coated with 1:1,000 dilution of anti-Alt a 1 in carbonate buffer (15–24 hours at 20–25°C) were incubated with serial dilutions of test samples and Alt a 1 standard, followed by detection of bound antigen with biotinylated anti-Alt a 1, avidin-alkaline phosphatase (Zymed, South San Francisco, California), and

p-nitrophenyl phosphate (Amresco, Solon, Ohio). Allergen-specific ELISA methods for German cockroach allergens Bla g 1 and Bla g 2 were performed using allergen-specific mouse monoclonal capture antibodies, reference antigens, and rabbit polyclonal probe antibody preparations obtained from Indoor Biotechnologies, and conditions comparable to those recommended by this manufacturer (Immulon 2 microplates, Thermo Electron Corp., Milford, Massachusetts; goat anti-rabbit IgG-alkaline phosphatase, Sigma, St. Louis, Missouri). Standard curves for these assays were constructed using absorbance changes over specified time periods (reaction rates) and log concentrations of reference antigens. Linear regression analyses identified the reference range exhibiting the highest correlation coefficient, with mean test sample values calculated using all absorbances falling within those of the selected references.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed with 12% polyacrylamide gels under nonreducing conditions, as described previously.^{7,11} Immunoblots were prepared and developed using methods detailed in previous publications.^{7,8,12}

Statistical analyses

Allergen recoveries for extract mixtures and controls were analyzed by two-sample *t* tests assuming equal variances and means, with significance achieved for data comparisons yielding two-tailed (two-sided) *P* values below .05.

Human subject participation was not required to perform these experiments; thus, this study was exempt from review by an institutional board or human subjects protection committee.

Results

Alternaria and German cockroach extract controls

Accurate determinations of the allergenic activities of *Alternaria* and German cockroach extracts in SCIT formulations are dependent on recovery of moderate to high activity levels in single-extract control samples at comparable glycerin concentrations. These values define the stabilities of target allergens independent of the effects of mixing with other fungal or insect extracts. Substantial reductions in control sample reactivity can limit the range and reliability of responses with corresponding extract mixtures. *Alternaria* control samples employed in this study displayed favorable recoveries (50% or higher) of multi-allergen IgE-binding potencies (relative to freshly prepared reference solutions of identical composition) and specific (major) allergen Alt a 1 concentrations (relative to previously determined values for the same test solutions) at all time points and glycerin levels examined. In contrast, German cockroach controls exhibited much lower recoveries of multi-allergen potencies (4–16% at 10% glycerin after 2–12 months, 34–42% at 25% glycerin after 4–12 months) compared with concentrations of specific allergen Bla g 1 (>70% at 10–25% glycerin after 2–12 months) and Bla g 2 (>50% at 10–25% glycerin after 2–12 months) at glycerin levels below 50%. These results affected the interpretations of potency values for cockroach extract mixtures at 10 to 25% glycerin concentrations, as detailed later.

Alternaria extract mixtures

Alternaria extracts exhibited favorable recoveries of IgE-binding potency after mixing with many fungal or insect extracts at 10 to 50% glycerin levels and storage for up to 12 months at 2°C to 8°C (Fig 1). IgE binding to *Alternaria* extract was reduced significantly when combined with fire ant extract at 10% glycerin (*P* < .00001) and 25% glycerin (*P* = .017). At 50% glycerin, these mixtures produced near-complete recoveries of IgE binding activity. Specific allergen analysis revealed no major changes in Alt a 1 concentration

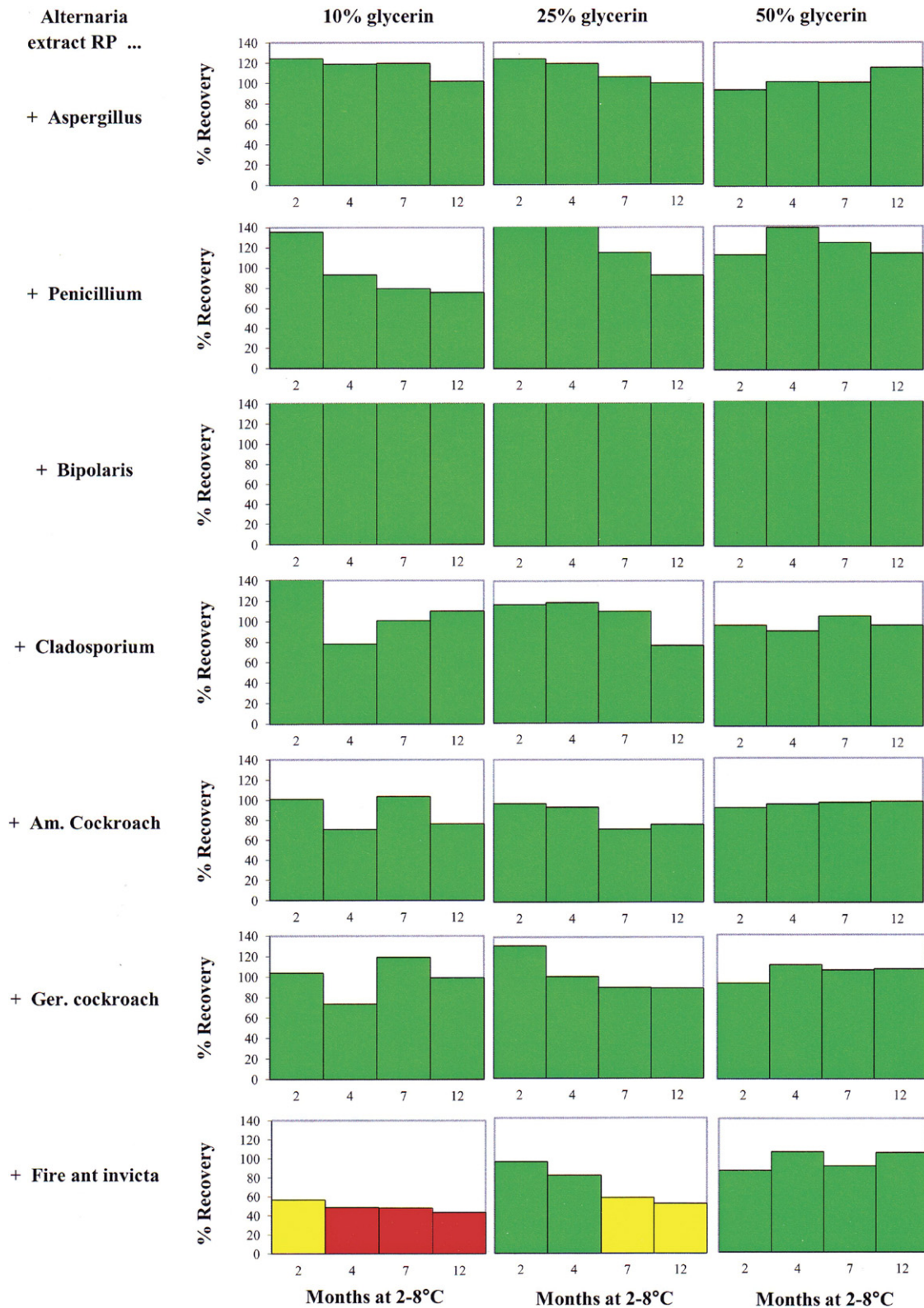


Fig. 1. Recoveries of IgE ELISA inhibition relative potency in *Alternaria* extracts at 10% glycerin, 25% glycerin, or 50% glycerin concentrations (left, center, and right columns, respectively) after mixing with other fungal or insect extracts (top row to bottom row) and storage for up to 12 months at 2 to 8°C. Percent recovery of IgE relative potency values at each time point are expressed relative to that of the *Alternaria* extract control sample at the same glycerin concentration. Recoveries greater than 70%, between 50% and 70%, and below 50% of the *Alternaria* extract control sample values at the same time point are shaded in green, yellow, and red, respectively.

in fire ant mixtures (contrary to IgE potency results), but significant reductions in Alt a 1 levels were observed after mixing with American cockroach extracts at 10 to 25% glycerin (Fig 2, $P < .00001$). These changes were not apparent in IgE potency assessments, and

they remained significant even at elevated (50%) glycerin levels ($P < .00001$). Alt a 1 recoveries were favorable in mixtures of *Alternaria* and all other fungal and insect extracts in this study (including German cockroach) at 10 to 50% glycerin for up to 12

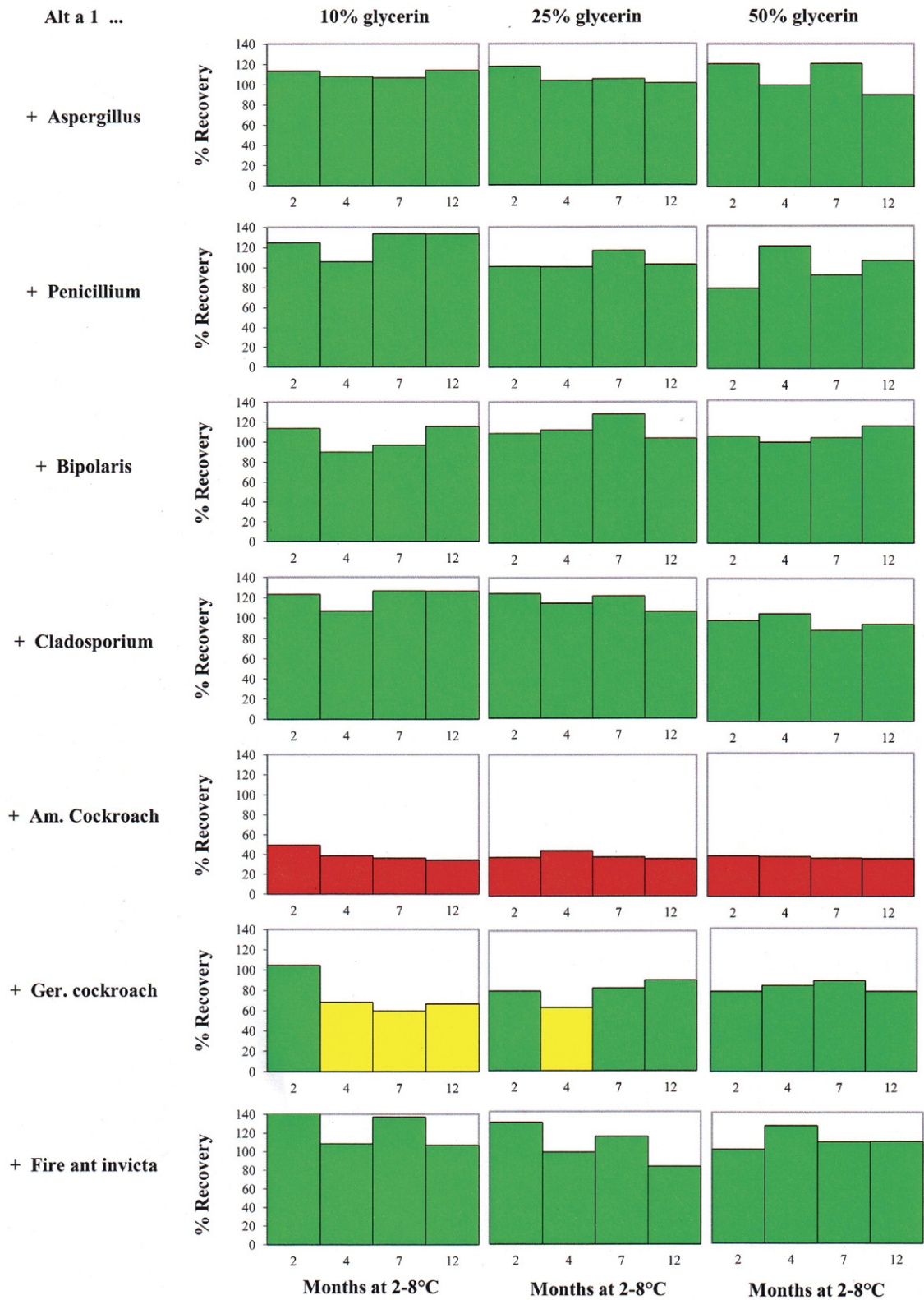


Fig. 2. Recoveries of Alt a 1 ELISA concentrations in *Alternaria* extracts at 10% glycerin, 25% glycerin, or 50% glycerin concentrations (left, center, and right columns, respectively) after mixing with other fungal or insect extracts (top row to bottom row) and storage for up to 12 months at 2°C to 8°C. Alt a 1 values are reported in microgram per milliliter concentrations from direct testing. Recoveries greater than 70%, between 50% and 70%, and below 50% of the *Alternaria* extract control sample values at the same time point are shaded in green, yellow, and red, respectively.

months at 2 to 8°C. Elevated (>140%) recoveries of IgE-binding activity in *Alternaria*–*Bipolaris* mixtures resulted from cross-reactivities between allergens other than Alt a 1 in these fungal extracts. IgE immunoblot analyses of *Alternaria* mixtures and controls stored

at 2 to 8°C for 7 months, as well as rabbit anti-Alt a 1 IgG immunoblots examined after 1 to 11 months at these temperatures, also produced no major changes in band patterns or intensities (data not shown).

German cockroach extract mixtures

German cockroach extracts mixed with fungal or insect products resulted in recoveries of multi-allergen IgE-binding potency ranging from near-complete to very low (less than 20% of freshly prepared controls), as illustrated in Figure 3. Values for extract combinations at 10% glycerin were displayed but not highlighted in color because of the uncertain accuracy of these results owing to the low recoveries of corresponding German cockroach control samples under the same conditions. Low recoveries also were observed for 25% glycerin controls at 7 and 12 months, which could affect the reliability of data from extract mixtures at these conditions. *Penicillium* extract ($P = .012$) and, to a less-significant degree, *Aspergillus* extract ($P = .090$) compromised German cockroach potencies at 25% glycerin during storage for up to 12 months at 2 to 8°C, with minor changes also found at 50% glycerin after 7 to 12 months. *Alternaria* or *Bipolaris* extracts were more compatible with German cockroach allergens but also led to allergen degradation over time at lower (10–25%) glycerin levels. These changes in multi-allergen potency were not detected in assays for specific allergens Bla g 1 and Bla g 2 (Figs 4 and 5). Reductions in Bla g 1 activity that were observed for several mixtures after 4 months were not found at later time points. Immunoblots of German cockroach mixtures and controls conducted after storage for 4 months at 2 to 8°C produced only minor changes at 10% glycerin (limited band patterns under these conditions), but numerous band pattern modifications were detected in mixtures with *Penicillium* and *Aspergillus* extracts at 25 to 50% glycerin levels (data not shown), consistent with the abilities of these fungal products to disrupt IgE binding to multiple German cockroach allergens in quantitative potency assays (Fig 3).

Discussion

Knowledge of the interactions between allergens and other constituents in extract mixtures can lead to effective and safe SCIT vaccines via preparation and delivery of more consistent and predictable allergen doses. Practice parameter updates provide guidelines for preparing patient formulations designed to deliver stable, compatible allergens at effective dose concentrations.² Numerous studies have demonstrated the presence of active hydrolytic enzymes in extracts of whole-body insects and cultured fungal organisms, supporting the separation of these extracts from low-protease or protease-susceptible products (pollen, dust mites, mammalian allergens, others) in patient treatment vials.^{2,4–10} However, only limited information, mostly anecdotal, exists to confirm the stabilities of allergens within high-protease extract mixtures.

The current study was performed to determine the stabilities and mixing compatibilities of fungal–insect extract combinations representative of those used for SCIT injections. The extracts studied are among the most allergenically important sources of fungal and insect aeroallergens, and the target allergens (*Alternaria*, German cockroach) are also closely associated with severe asthma exacerbations.^{13,14} A broad spectrum of allergens has been identified in these extracts, including major allergens in the 2 target extracts (*Alternaria* Alt a 1, German cockroach Bla g 1, and Bla g 2). In-house human serum pools from patients with IgE-mediated sensitivities to *Alternaria* or cockroach were created to develop ELISA inhibition assays for multi-allergen potency similar in configuration and performance to the methods currently used to standardize grass pollen and dust mite extracts in the United States.^{15,16} Specific allergen ELISA assays for Alt a 1, Bla g 1, and Bla g 2 were also performed.^{17,18} Recognizing that specific-allergen and multi-allergen methods employ different antibody reagents and configurations (sequential steps), which can directly affect their abilities to detect changes in allergen structures, is important.^{15,19–21} The tar-

get interactions responsible for allergen capture and quantitation range from a single IgG binding site on 1 allergenic molecule to multiple IgE binding sites on numerous allergens. Because the epitopes recognized by allergen-specific IgG antibodies may not coincide with some of those bound by the human IgE antibodies in allergic serum pools, structural changes to these allergens may not be detected proportionately by these methods, and modifications to nonhomologous regions on other allergens in the extract would not be accounted for in results from single-allergen tests. Multiple methods with diverse molecular targets and binding specificities were included in the present study to circumvent the potential uncertainties related to adoption of a single (or more limited) analytical approach with undetermined or limited clinical significance.

Endogenous proteolytic enzymes can impact allergenic activities directly (cleavage of critical antibody-binding sequences), indirectly (conformational changes induced by protease actions at remote locations), or in a combination of these effects. In individual extracts, the allergens and enzymes (some of which could be allergenic) are derived from the same source materials, and any degradative effects impacting allergen activities are apparent in single-extract control samples such as those included in the current study. Based on results from these samples, German cockroach extract potencies were found to be very sensitive to autodigestion by endogenous proteases at low to moderate (10–25%) glycerin concentrations, with *Alternaria* extract potencies exhibiting much higher stability, even at low (10%) glycerin levels. These differences can result from product-to-product variations in protease concentrations or specific activities, the presence and levels of protease inhibitors (natural or unintentional), selective effects of enzyme action on allergenic sequences or remote (conformational) structural regions, or other factors.

In mixtures of protease-rich extracts, these interactions, and their possible harmful effects, are multiplied and more difficult to predict compared with individual extracts. In the 2-component mixtures evaluated here, interactions between allergens from 1 product and proteases from the other were examined using methods specific for 1 of the 2 extracts, and for both target extracts in *Alternaria*–German cockroach mixtures. Allergen compatibilities in the companion extracts were not determined because of a lack of appropriate, specific antibody reagents or test methodologies for these products.

For both *Alternaria* and German cockroach, distinctive patterns of extract compatibility were observed. Interestingly, these extracts exhibited favorable specific-allergen and multi-allergen stabilities after mixing with other extracts from within the same phylogenetic group (*Alternaria* with other fungi from the class Deuteromycetes, German cockroach with other insects from the class Insecta) at 10 to 50% glycerin concentrations when stored for up to 12 months at 2 to 8°C. In mixtures of extracts from the 2 taxonomic groups, however, assay-dependent susceptibilities were found. *Alternaria* extract potencies were compromised by mixing with fire ant extracts, but Alt a 1 activities were not affected in these mixtures. Conversely, Alt a 1 binding was reduced significantly in mixtures with American cockroach and, to a lesser degree, German cockroach, but no reductions in multi-allergen potency of *Alternaria* extracts were detected in the same samples. The inability of glycerin at high (50%) concentrations to stabilize Alt a 1 binding to rabbit antibodies in American cockroach mixtures was unusual, relative to all of the other extract combinations examined in this and previous studies conducted in our laboratory, and is under further investigation.^{7,8} Cross-reactivities between *Alternaria* and *Bipolaris* extract components affected allergen recoveries in multi-allergen IgE-binding assays but not specific allergen Alt a 1 assessments. Retention of the IgE- and IgG-binding properties of Alt a 1 molecules (31 kD) was also observed on immunoblots using extract

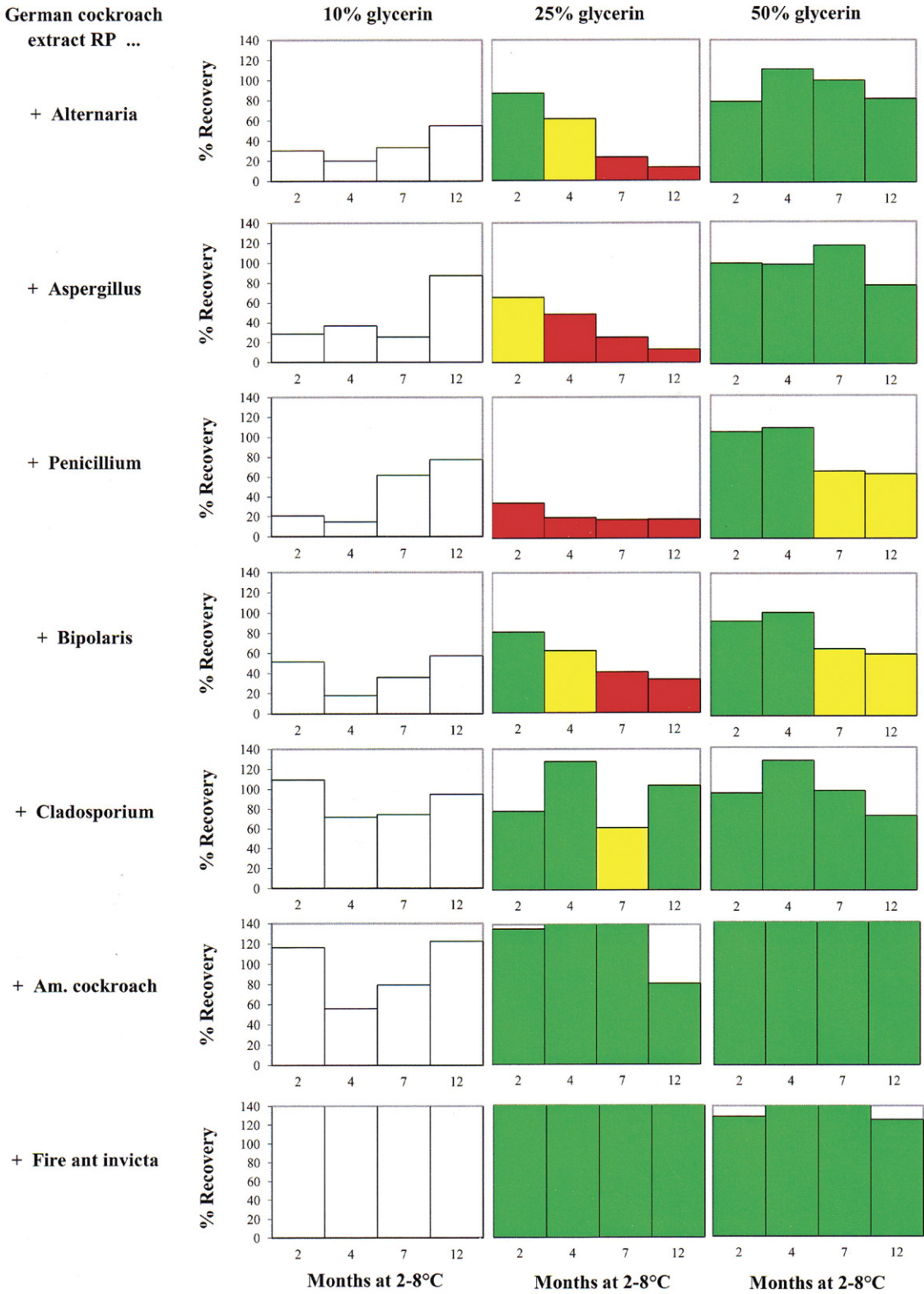


Fig. 3. Recoveries of IgE ELISA inhibition relative potency in German cockroach extracts at 10% glycerin, 25% glycerin, or 50% glycerin concentrations (left, center, and right columns, respectively) after mixing with other fungal or insect extracts (top row to bottom row) and storage for up to 12 months at 2°C to 8°C. Percent recovery of IgE relative potency values at each time point are expressed relative to that of the German cockroach extract control sample at the same glycerin concentration. Recoveries for 10% glycerin samples were not highlighted in color because of the uncertain accuracy of these values relative to the low recoveries for corresponding control samples (Fig 2). Recoveries greater than 70%, between 50% and 70%, and below 50% of the German cockroach extract control sample values at the same time point are shaded in green, yellow, and red, respectively, for extract mixtures at 25% glycerin and 50% glycerin.

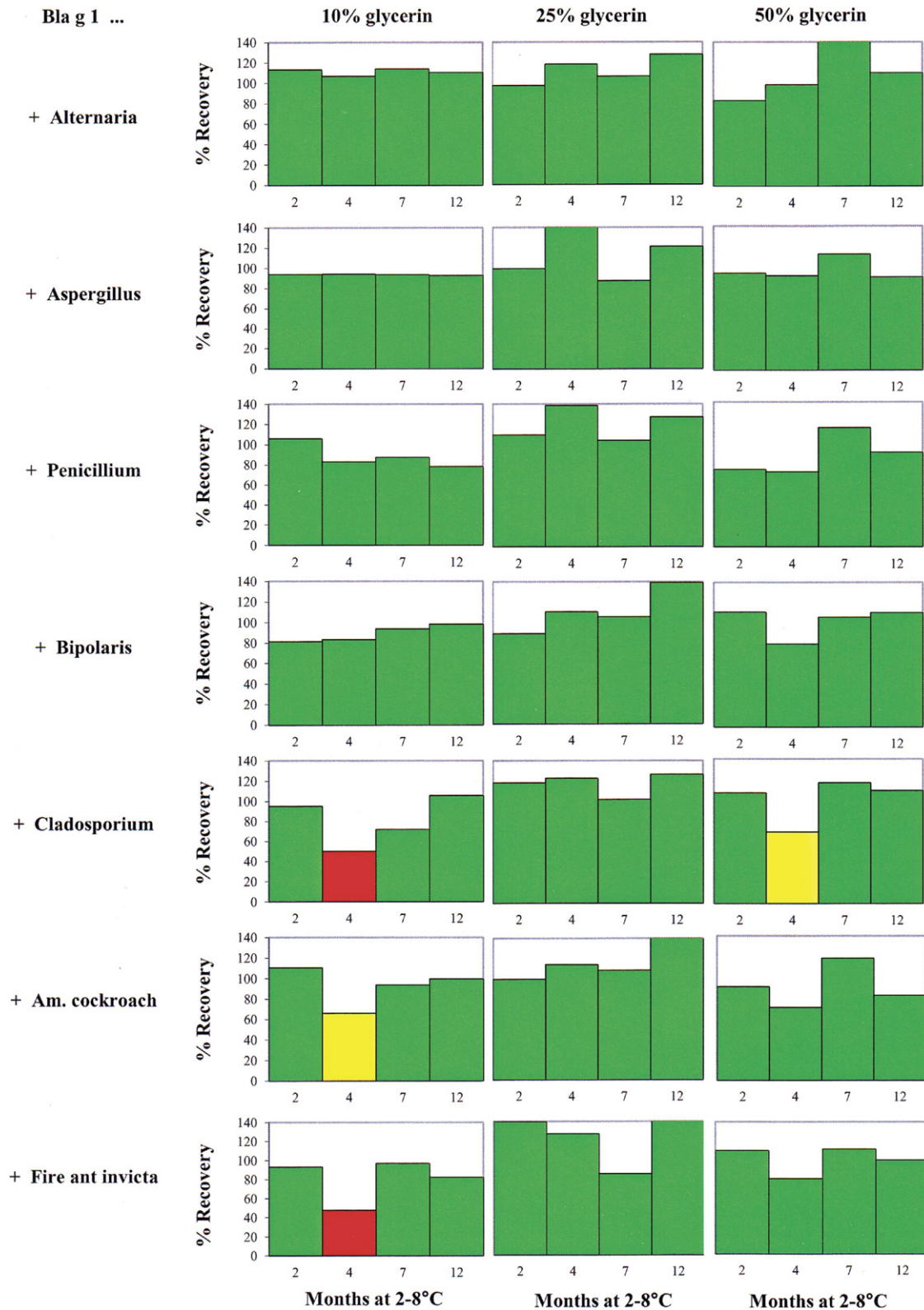


Fig. 4. Recoveries of Bla g 1 ELISA concentrations in German cockroach extracts at 10% glycerin, 25% glycerin or 50% glycerin concentrations (left, center, and right columns, respectively) after mixing with other fungal or insect extracts (top row to bottom row) and storage for up to 12 months at 2°C to 8°C. Bla g 1 values are reported in Unit/mL concentrations from direct testing. Recoveries greater than 70%, between 50% and 70%, and below 50% of the German cockroach extract control sample values at the same time point are shaded in green, yellow, and red, respectively.

mixtures subjected to heat and detergent conditions known to disrupt 3-dimensional protein conformations. German cockroach extracts retained high levels of Bla g 1 and Bla g 2 activities in all extract mixtures tested, but multi-allergen IgE-binding activities

were diminished significantly after mixing with *Penicillium* extract, prominently but less significantly after mixing with *Aspergillus* extract, and to a more limited degree after combining with *Alternaria* or *Bipolaris* extracts. IgE immunoblots confirmed that degra-

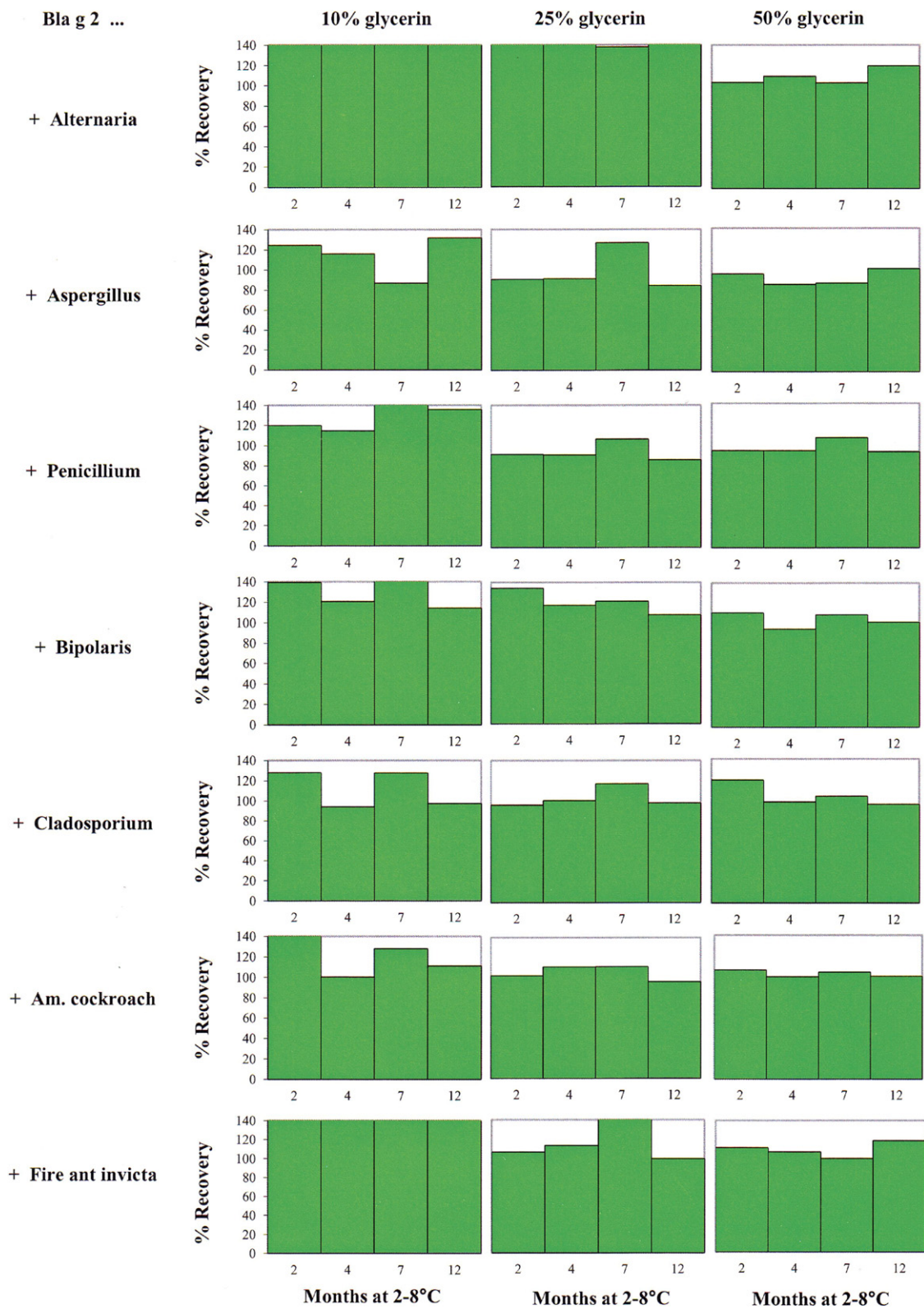


Fig. 5. Recoveries of Bla g 2 ELISA concentrations in German cockroach extracts at 10% glycerin, 25% glycerin, or 50% glycerin concentrations (left, center, and right columns, respectively) after mixing with other fungal or insect extracts (top row to bottom row) and storage for up to 12 months at 2 to 8°C. Bla g 2 values are reported in microgram per milliliter concentrations from direct testing. Recoveries greater than 70% of the German cockroach extract control sample values at the same time point are shaded in green.

dation of cockroach extract proteins was occurring in the same *Penicillium* and *Aspergillus* mixtures that displayed stable Bla g 1 and Bla g 2 concentrations.

Based on a conservative view of these results (any test method demonstrating changes in allergen activities), additions of fungal

and insect extracts to separate patient vials may be needed to stabilize *Alternaria* and cockroach allergens for SCIT injections. The presence of elevated glycerin levels, or storage of these solutions for shorter periods, could alleviate some, possibly most, of these incompatibilities. For some patients, the retention of extract activ-

ities demonstrated by these data may correlate closely with their in vivo activities and specificities, such that little or no change in the essential activities of these extracts would occur after mixing with other high-protease products. The observations from the present study of 3-fold to 10-fold variations in allergen extract recoveries for samples assayed with different test procedures validated the decision to evaluate multiple immunochemical methods and help to establish an analytical foundation for future investigations with target extracts that contain a diverse repertoire of major and minor allergenic components.

This study was limited in several ways. Extract lots were obtained from only 1 manufacturer (Greer Laboratories), and human serum pools were constructed from a relatively small number of allergic donors. In vivo assessments of these extract mixtures should also be conducted, particularly for those combinations exhibiting discrepant specific-allergen and IgE-binding extract recoveries. In addition, extract mixtures containing 3 or more high-protease extracts typical of those used regularly for SCIT should be evaluated. The data presented here may serve as a starting point for future studies with more complex extract formulations.

In summary, inclusion of quantitative assays for both single (major) allergens and multiple IgE-binding constituents, and qualitative tests for IgE-binding patterns, each with distinct allergen-antibody binding specificities and assay susceptibilities, provided a comprehensive assessment of the stability and compatibility of allergens in high-protease extract mixtures, and identified several cross-taxonomic extract combinations (*Alternaria* with insects, German cockroach with fungi) that could compromise the stability of allergens in SCIT mixtures.

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